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UTILITY PATENT APPLICATION TRANSMITTAL	
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First Inventor or Application Identifier Mytych et al.	
Title Methods and Reagents for the Detection of Antibodies etc	
Express Mail Label No. EL226883618US	

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17. CORRESPONDENCE ADDRESS					
<input type="checkbox"/> Customer Number or Bar Code Label 24265 or <input checked="" type="checkbox"/> Correspondence address below					
(Insert Customer No. or Attach bar code label here)					
Name	Donald W. Wyatt				
	Patent Dept. K-6-1, 1990				
Address	Schering-Plough Corporation				
	2000 Galloping Hill Road				
City	Kenilworth	State	New Jersey	Zip Code	07033-0530
Country	USA	Telephone	908-298-2902	Fax	(908) 298-5388

Name (Print/Type)	Donald W. Wyatt	Registration No. (Attorney/Agent)	40879
Signature		Date	August 22, 2000

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PATENT APPLICATION

**METHODS AND REAGENTS FOR THE DETECTION
OF ANTIBODIES TO ADENOVIRUS**

INVENTORS:

Daniel T. Mytych, a citizen of the United States, residing at
3162 Saddleback Court, Thousand Oaks, CA 91360, U.S.A.

Steven J. Swanson, a citizen of the United States, residing
at 4318 Timberdale Road, Moorpark, CA 93021, U.S.A.

ASSIGNEE: **Schering Corporation**

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Date of Deposit: August 22, 2000

Donald W. Wyatt
Schering-Plough Corporation
Patent Department, K-6-1, 1990
2000 Galloping Hill Road
Kenilworth, New Jersey 07033-0530
Telephone No.: (908) 298-2902
Facsimile No.: (908) 298-5388

METHODS AND REAGENTS FOR THE DETECTION OF ANTIBODIES TO ADENOVIRUS

5 This application claims the benefit of United States Provisional Application
No. 60/150,622 filed August 25, 1999.

Throughout this disclosure, various publications, patents and patent applications are
referenced. The disclosures of these publications, patents and patent applications are herein
incorporated by reference.

10 **Field of the Invention**

The present invention pertains to methods and reagents for the detection of
antibodies that bind to adenovirus. In preferred embodiments, the present invention is
directed to detecting antibodies to adenovirus 5. The usefulness of the present
invention includes, but is not limited to, identifying patients likely to have an immune
15 response to gene therapy, vaccines to prevent or treat adenovirus infection, therapeutics
to induce passive immunity to adenovirus infection and the like.

Background

Prospects for gene therapy to correct genetic disease or to deliver therapeutic
molecules depend on the development of gene transfer vehicles that can safely deliver
20 exogenous nucleic acid to a recipient cell. To date, most efforts have focused on the
use of virus-derived vectors that carry a heterologous gene (transgene) in order to
exploit the natural ability of a virus to deliver genomic content to a target cell.

For example, despite their reputation as major pathogenic agents that lead to
numerous infectious diseases, adenoviruses (and particularly, replication-deficient
25 adenoviruses) have attracted considerable recognition as highly effective viral vectors
for gene therapy. Adenoviral vectors offer exciting possibilities based on their high
efficiency of gene transfer, substantial carrying capacity, and ability to infect a wide
range of cell types. Due to these desirable properties of adenoviruses, recombinant
adenoviral vectors have been used for the cell-targeted transfer of one or more
30 recombinant genes to diseased cells or tissue in need of treatment. In fact, adenovirus-
based vectors offer several advantages, including tropism for both dividing and non-
dividing cells, minimal pathogenic potential, ability to prepare vector stocks at high
titer, and the potential to carry large DNA inserts. To date, genes that have been
expressed by adenoviral vectors include p53, dystrophin, erythropoietin, ornithine

transcarbamylase, adenosine deaminase, interleukin-2 and α -antitrypsin. Examples of adenovirus vectors can be found in U.S. Patent No. 5,585,362 to Wilson et al., U.S. Patent No. 5,824,544 to Armentano et al., and U.S. Patent No. 5,846,782 to Wickham et al.

One barrier to successful gene transfer by viral vectors to patient hosts is the immune response of the host to the introduction of the virus. In terms of the general structure of an adenovirus, under the electron microscope, an adenovirus particle resembles a space capsule having protruding antennae. The viral capsid comprises at least six different polypeptides, including hexon, base and fiber proteins. The fiber, together with the hexon (Crawford-Miksza, L. and Schnurr, D. P., J. Virology, Mar 1996, pp.1836-1844), determine the serotype specificity of the virus, and also comprise the main antigenic determinants of the virus.

This ability of adenoviral fiber and hexon protein to act as targets for a host immune response hamper attempts at adenoviral-mediated gene therapy. Namely, following adenoviral vector re-administration to prolong the therapeutic response, neutralizing antibodies develop against the adenoviral fiber and/or hexon proteins, thus circumventing adenoviral gene delivery to host cells. As the therapy is expensive, it is extremely wasteful to utilize a viral vector for gene therapy in a patient host that will mount an immune response to the vector.

What is needed is an efficient method of determining the likelihood that a patient host's immune system will interfere with intended gene therapy using viral vectors.

Summary of the Invention

The present invention provides methods and reagents for detecting antibodies capable of binding to adenovirus. In one embodiment, the present invention provides a method for detecting antibodies capable of binding to adenovirus comprising: a) immobilization of one or more peptides capable of being bound by an anti-adenovirus antibody directly onto a flowcell of a sensorchip in a biosensor, b) obtaining a serum sample from a patient to be tested and contacting said serum sample with the immobilized peptide, and c) measuring binding of antibodies to the immobilized peptide by means of the biosensor. In preferred embodiments, more than one peptide is utilized and are directly immobilized, each on its own separate flowcell.

In another embodiment, the present invention provides a method for detecting antibodies capable of binding to adenovirus, comprising a peptide selected from the group consisting of: AATALEINLEEEDDDNEDEVDEQAEQQKTHVF-Amide, IGVEGQTPKYADK-Amide, YETEINHAAGRVLKK-Amide, GILVKQNGKLESQ-Amide, STTEATAGNGDNLTPKV-Amide, MPTIKEGNSRELMG-Amide, VINTETLTKVKPKTGQENGWEKDATEFSK-Amide, or peptides having substantial sequence identity thereto. While not limited to a specific method of detection, in one embodiment the method of detecting comprises an ELISA system.

In yet another embodiment, the present invention provides a composition of matter comprising CKGKG or a peptide having substantial sequence identity thereto, and their use in a biosensor based assay to detect antibodies.

While certain embodiments of the present invention is not limited to specific peptides, in preferred embodiments the peptide is capable of being bound by antibodies specific to adenovirus 5. Examples of such peptides include those described above, as well as the following: CKGGAATALEINLEEEDDDNEDEVDEQAEQQKTHVF-Amide, CKKGIGVEGQTPKYADK-Amide, CKKGYEINHAAGRVLKK-Amide, CKKGILVKQNGKLESQ-Amide, CKKGSTTEATAGNGDNLTPKV-Amide, CKKGMPPTIKEGNSRELMG-Amide, CKKGVINTETLTKVKPKTGQENGWEKDATEFSK-Amide, or peptides having substantial sequence identity thereto.

In another embodiment, the present invention encompasses peptides that correspond to family reactive determinants of hexon and penton. This includes peptides that cross-react with similar antigens of other viral vectors.

The present invention is also not limited by the sample to be analyzed for antibodies. In one embodiment, the sample is human serum. In a preferred embodiment, the serum sample is simultaneously contacting with a plurality of immobilized peptides. Likewise, it is preferred that when more than one peptide is used, at least one of the plurality of peptides is capable of being bound by antibodies specific to adenovirus 5.

While not limited by the desired results of an assay, in preferred embodiments, the amount of antibody that binds to each peptide is directly proportional to the response units that are reported by the biosensor.

Finally, although the present invention is not limited by the equipment used; in a preferred embodiment, it is a BIACORE 2000™ biosensor.

(The above sequences are listed using standard one-letter amino acid symbols; *see e.g.*, Lehninger, *Principles of Biochemistry*, Worth Publishers, Inc. 6th Ed. 1988, p.96).

Definitions

An "antibody" includes, but is not limited to, immunoglobulin molecules and immunologically active portions of immunoglobulin molecules such as portions containing a paratope (i.e., an antigen binding site). In particular, an antibody preferably can be a bispecific antibody, i.e., having one paratope directed to an epitope of the chimeric fiber protein, and another paratope directed to an epitope of a cell surface-binding site.

A "vector" according to the invention is a vehicle for gene transfer, as that term is understood by those skilled in the art. Three types of vectors encompassed by the invention are plasmids, phages, and viruses. Plasmids, phages, and viruses can be transferred to a cell in their nucleic acid form (e.g., via transfection). In comparison, phages and viruses also can be transferred with the nucleic acid in a "capsular" form. Hence, the vectors (e.g., capsular form) that can be employed for gene transfer are referred to herein generally as "vectors", with nucleic acid forms being referred to more particularly as "transfer vectors". However, transfer vectors also are vectors within the context of the invention.

"Substantial sequence identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more. Preferably, residue positions that are not identical differ by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to effect the properties of a protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid. In addition, substantial sequence identity encompasses amino acids or chemicals that create a similar structure. For example, linear, cyclic, or constrained peptides.

Detailed Description of the Invention

The present invention provides methods and reagents for detecting antibodies to adenovirus. In one embodiment, the present invention provides peptides for detecting anti-adenovirus antibodies. In another embodiment, the present invention provides novel peptides for detecting anti-adenovirus antibodies in a biosensor-based assay. The

present invention is not limited to the serotype of the adenovirus capable of being bound by the antibodies detected. In a preferred embodiment, however, the serotype is adenovirus 5 (AV5).

The present invention is also not limited to the specific type of assay used to detect the anti-adenovirus antibodies.

In one embodiment, the present invention contemplates the use of an enzyme-linked immunosorbant assays (ELISA). An ELISA assay, in general, is performed by binding a reference reagent (antigen) to a solid phase support. Samples to be tested are mixed with a labeled reagent, then reacted with the bound reference reagent. The reagents then undergo a series of dilution, incubation, and washing steps in order to separate bound and free reagents. The process concludes with a detection step, compatible with the type of label used, designed to indirectly measure the amount of antibody (or antigen) in the samples tested. For example, a commercially available enzyme-linked immunosorbant assay (ELISA) to detect enteric adenoviruses based on a polyclonal antibody to enteric adenovirus hexon protein is available (Adeno-Type 40/41 EIA, Cambridge Bioscience, Cambridge, MA).

In another embodiment, the present invention contemplates the use of ORIGEN electrochemiluminescence (ECL) detection system (Igen, Gaithersburg, MD). Briefly, the ECL system utilizes strepavidin-coated paramagnetic beads to capture and detect immune complexes formed between antigen bound to a biotin-conjugated antibody.

The present invention also contemplates the use of a biosensor-based assay. The biosensor used in the present invention is preferably a BIACORE 2000™ from Pharmacia (Uppsalla, Sweden). The BIACORE 2000™ operates on a principle of surface plasmon resonance and allows for high throughput analyses. (*See, e.g.,* Hodgson, *Bio/Technology* vol. 12, Jan. 1994 for a review of biosensors). Other biosensors, *e.g.,* a BIACORE™ X (Biacore 1000™; Biacore 3000™) from Pharmacia or an IAsys biosensor from Fisons, could be used in connection with the present invention. In analyzing the results of the assay, it is preferred that the amount of antibody that binds to each peptide is directly proportional to the biosensor signal (*e.g.,* the response units that are reported by the BIACORE 2000™ biosensor).

Among other advantages, use of a biosensor assay in the present invention provides an assay that is suited for detecting lower affinity antibodies (Obenauer-Kutner et al., *Journal of Immunological Methods*, 1997, 206:25-33; Swanson et al., *Dev. Biol. Stand. Basel*, Karger, 1999, 97:135-147). Methods employing ELISAs have

difficulty in this regard because ELISAs require multiple incubation steps followed by wash cycles during which lower affinity antibodies can wash away. There is also a need for an assay that is less labor intensive than the methods utilizing an ELISA format. Finally, there is a need to significantly reduce analysis time, preferably to less than 10 minutes/sample, to allow for high throughput analyses. As mentioned above, ELISAs require multiple incubation steps followed by wash cycles, and therefore the materials used in the assay cannot be regenerated for analysis of subsequent samples.

While the present invention is not limited to specific peptides for detection of adenovirus, preferred peptides for use in the methods of the present invention are comprised of one or more of the sequences below (or peptides having substantial sequence identity thereto):

- a) Peptide SEQ ID NO: 1 (HVR1 of AV5),
AATALEINLEEEEDDDNEDEVDEQAEQQKTHVF-Amide;
- b) Peptide SEQ ID NO: 2 (HVR2 of AV5),
IGVEGQTPKYADK-Amide
- c) Peptide SEQ ID NO: 3 (HVR3 of AV5),
YETEINHAAGRVLKK-Amide
- d) Peptide SEQ ID NO: 4 (HVR4 of AV5),
GILVKQQNGKLESQ-Amide
- e) Peptide SEQ ID NO: 5 (HVR5 of AV5),
STTEATAGNGDNLTPKV-Amide
- f) Peptide SEQ ID NO: 6 (HVR6 of AV5),
MPTIKEGNSRELMG-Amide
- g) Peptide SEQ ID NO: 7 (HVR7 of AV5),
VINTETLTKVKPKTGQENGWEKDATEFSDK-Amide

The above sequences are described in Crawford-Mikszta et al., J. Vir. 70:1836, 1996, Toogood et al., J. Gen. Vir. 73:1429, 1992, and Kinloch et al., J. Biol. Chem 259:6431, 1984. These peptides, however, are reported as part of conformational proteins. Surprisingly, peptides HVR1 (SEQ ID NO: 1) and HVR6 (SEQ ID NO: 6) have been identified as part of internalized loops within the hexon protein that are not even exposed to the external surface (Haase & Pereira, J. Immunol. 108:633-636, 1972; Kjellen & Pereira, J. Gen. Vir. 2:177-185, 1968; Mautner & Wilcox, J. Gen. Vir. 25:325-336, 1974; Norrby, Virology 37:565-576, 1969; Wilcox & Mautner, J. Immunol. 116:19-24, 1976). It would be expected, therefore, that if these peptides are

suitable as targets for antibody binding, that the target of antibody binding is part of the protein's overall secondary and tertiary structure. As such, it is surprising that these short linear peptides, even within the internal loop, are suitable for detecting neutralizing viral antibodies. Furthermore, it is surprising to find serotype-specificity within peptides that include conserved sequences between serotypes.

When a peptide is used in a biosensor-based assay, it is preferred that a positively charged leader sequence be added next to a cysteine at the beginning of the peptide. This leader sequence interacts with the net negatively charged solid support of the biosensor system to enhance the immobilization of the peptide to the carboxymethyl dextran support surface. While not limited to a particular leading sequence, the first position must be cysteine (C) and the second position can be either lysine (K), arginine (R) or histidine (H), in a preferred embodiment, the leading sequence is CKGKG (SEQ ID NO: 8) (or peptides having substantial sequence identity thereto). This results in preferred peptides for use in a biosensor-based assay of (or peptides having substantial sequence identity thereto):

- a) Peptide SEQ ID NO: 9 (HVR1 of AV5),
CKGKGAATALEINLEEDDDNEDEVDEQAEQKTHVF-Amide;
- b) Peptide SEQ ID NO: 10 (HVR2 of AV5),
CKGKGIGVEGQTPKYADK-Amide
- c) Peptide SEQ ID NO: 11 (HVR3 of AV5),
CKGKGYETEINHAAGRVLKK-Amide
- d) Peptide SEQ ID NO: 12 (HVR4 of AV5),
CKGKGILVKQQNGKLESQ-Amide
- e) Peptide SEQ ID NO: 13 (HVR5 of AV5),
CKGKGSTTEATAGNDNLTPKV-Amide
- f) Peptide SEQ ID NO: 14 (HVR6 of AV5),
CKGKGMPITKEGNSRELMG-Amide
- g) Peptide SEQ ID NO: 15 (HVR7 of AV5),
CKGKGVINTETLTKVKPKTGQENGWEKDATEFSDK-Amide

When viral vectors are used to deliver gene therapy to a patient, the patient's immune reaction to the viral vector has a significant impact on the success of the therapy. For example, if a patient's immune system produces antibodies to the viral

vector, the vector may be inactivated before it has properly delivered the therapeutic gene to the patient's cells.

Therefore, information about a patient's immune system and their antibodies against viral vectors is important. As an example, the level of high-concentration and/or high-affinity antibodies will have a direct impact on therapy. Moreover, the level of low-concentration and/or low-affinity antibodies is also an important factor to consider. Even though the amount of low-affinity antibodies may have a limited impact on the early stages of therapy, the data obtained may predict the development of increased quantities of antibodies or additional higher-affinity antibodies. Furthermore, the ratio of high-affinity to low-affinity antibodies is a valuable indicator that permits a medical provider to design and monitor a patient's therapy.

When the methods of the present invention are used, it is possible to detect low-concentration and/or low-affinity antibodies and differentiate them from the high concentration and/or high affinity antibodies. For example, using a system such as the BIACORE 2000™ to detect antibodies provides information on the amount of antibodies that can bind to an antigen, whether they are high- or low-affinity antibodies. This information can be compared to detection of antibodies under more stringent conditions (e.g., ELISA) that detect high-concentration and high-affinity antibodies. Thus an immune status profile can be generated that evaluates the amount of total antibodies, and whether they are of high-affinity and/or high concentration antibodies or low-affinity and/or low concentration antibodies.

The present invention further contemplates the preparation and use of a vaccine composition for the treatment of human adenovirus infection, including AV5. The preparation of such a vaccine is accomplished by utilization of at least one of the above peptides. This can be accomplished by utilization of at least one of the above peptides. This can be accomplished by genetic engineering of at least one of the above peptides and expressing at least one of these proteins in suitable vector/host cell systems such as bacteria, yeast or any other suitable vector/host system. In another embodiment, the present invention is directed to a Type II vaccine which is a combination of an inactivated adenovirus and at least one of the above-listed proteins.

By vaccine is meant an agent used to stimulate the immune system of a living organism so that protection against future harm is provided. A number of viral polypeptide preparations derived from viral coats or envelopes have been suggested as possible active components for vaccine compositions. For example, U.S. Pat. No.

4,470,967 describes vaccine preparations which are made by complexing viral polypeptide with a lectin, the latter element acting as adjuvant. A number of references, (e.g., 4,344,935 or 4,356,169 or Morein, et al., J. Gen. Virol., 64: 1557-1569, 1983), utilize a method of preparing parainfluenza glycoprotein compositions in which the viral glycoprotein HN and F are solubilized with a detergent, to extract them from the viral envelope, followed by some method of phase separation in order to remove the detergent and lipids. The latter procedures produce a glycoprotein subunit that is not only substantially detergent-free, but also lipid-free. The latter type of highly purified glycoprotein is considered the preferred type of active agent for potential use of commercial vaccine. An alternative approach is the synthesis of peptides for use as a vaccine.

Recombinant DNA techniques for the preparation of recombinant adenovirus peptides for use in the preparation of vaccines are sufficiently well known and widespread so as to be considered routine. In very general and broad terms, a method for use herein consists of transferring the genetic material, or more usually part of the genetic material, of one organism into a second organism so that the transferred genetic material becomes a permanent part of (recombines with) the genetic material of the organisms to which it is transferred.

This usually consists of first obtaining a small piece of DNA from the parent organism either from a plasmid or a parent chromosome. A plasmid (also called an extrachromosomal element) is a hereditary unit that is episomal, (i.e.- physically separate from the chromosome of the cell). The DNA may be of any size and is often obtained by the action of a restriction endonuclease enzyme that acts to split DNA molecules at specific base-pair sites. In the present invention an adenovirus peptide gene can be synthesized based upon the sequence of the protein to be expressed.

DNA pieces may be transferred into a host cell by various means. For example, transformation wherein naked DNA is internalized into a cell from the external environment, oftentimes through artificially induced disruption of the cell membrane (e.g. by introduction of various chemical agents, such as calcium ions or lipids, by application of electric current, by extreme temperature changes or by microinjection). Other methods of gene transfer such as transduction are also suitable, wherein DNA is packaged within a phage such as a cosmid or viral vector.

Once DNA is in the host cell, it may continue to exist as a separate piece (generally true of complete transmitted plasmids) or it may insert into the host cell chromosome and be reproduced together with the chromosome during cell division.

Administration of a vaccine contemplated by the present invention to the patient (human or animal) may be by any known or standard techniques. These include oral ingestion, intestinal intubation, or broncho-nasal spraying. Other methods of administration, such as intravenous injection, that allow the carrier microbe to reach the human or animal's bloodstream may be acceptable when the carrier microbe is replication deficient.

The amount required will vary with the antigenicity of the gene product and need only be an amount sufficient to induce an immune response typical of existing vaccines. Routine experimentation will easily establish the required amount. Typical initial dosages of vaccine could be about 0.001-100 mg antigen/kg body weight, with increasing amounts or multiple dosages used as needed to provide the desired level of protection.

The pharmaceutical carrier in which the vaccine is suspended or dissolved may be any solvent or solid that is non-toxic to the inoculated animal and compatible with the carrier organism or antigenic gene product. Suitable pharmaceutical carriers include liposomes and liquid carriers, such as normal saline and other non-toxic salts at or near physiological concentrations, and solid carriers, such as talc or sucrose. Adjuvants, such as Freund's adjuvant, complete or incomplete, may be added to enhance the antigenicity via the bronchial tubes, wherein the vaccine is suitably present in the form of an aerosol. Booster immunizations may be repeated numerous times with beneficial results.

In another aspect, the present invention relates to a method of treating infectious diseases caused by adenovirus infection by introducing passive immunity. Passive immunization, as defined herein, refers to resistance (e.g., temporary or sustained protection against infection) based on giving preformed antibodies to a patient from an in vivo or in vitro source. The main advantage of passive immunization is the prompt availability of large amounts of antibodies against human adenoviruses that can be identified as described in the above embodiment of the present invention.

For example, the present invention also encompasses antibodies, either monoclonal or polyclonal, that are identified using the systems described above and are useful in the therapeutic control of infection by adenoviruses. Said antibodies can be

prepared by injecting mammalian species, e.g., human, horse, rabbit, sheep, mice, etc. with the peptides described above and then purifying said antibodies employing the detection systems contemplated and described herein.

In another embodiment, the present invention relates to the development of specific human or other eukaryotic (e.g., yeast, baculovirus, or Chinese hamster cells) polyclonal or monoclonal antibodies, as well as human-mouse chimeric polyclonal or monoclonal antibodies for administration in passive immunization against human adenoviruses. Such antibodies can be used as created in these systems, or they can be humanized. Methods of humanizing antibodies is described in U.S. Patents No.

5,597,710 and 5,705,154 to Dalie et al. and U.S. Patent No. 5,585,089 to Queen et al.

In addition, the generation of antibodies against the disclosed peptides can be used for commercial use. In one embodiment, these antibodies may be used as probes for various biochemical assays to detect adenovirus, more preferably AV5. In another embodiment, these antibodies may be used in kits to detect IR.

The present invention further contemplates the use peptides as probes to detect, by hybridization, cellular DNA from infected tissue (e.g. biopsy material) carrying integrated structural adenovirus DNA (i.e., DNA encoding one of the peptides described above). The probe can be DNA, cDNA, recombinant DNA or RNA.

In one particular embodiment of the present invention, the probes may be used for in situ hybridization. For example, patient specimens (tissue or tissue extracts) containing biopsy material are smeared onto a standard microscope slide, then fixed with an appropriate fixative. The DNA or RNA probe, which has been labeled (e.g. with biotin-avidin-enzyme) is added. The slide is then placed onto a heating block for one or two minutes to allow both the probe and the target nucleic acids to be separated from their complementary strand (if double stranded). Non-hybridized probe DNA or RNA is removed by gentle washing. After a suitable detection complex is added, hybridization is detected with a light microscope following formation of a colored compound. In other embodiments of the invention, the nucleic acid probe is labeled with a radioactive isotope or chemiluminescent tag. Alternatively, tissue to be tested may be lysed and DNA/RNA fixed to, nitrocellulose paper for example. Hybridization and DNA/RNA detection systems are well known in the art.

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

Example 1: MATERIALS AND METHODS**1.1 Equipment and Materials**

BIACORE 2000™ biosensor instrument, amine coupling kit (NHS/EDC), PDEA, sensorchip CM5 (research grade) and P-20 surfactant were all obtained from BIACORE™, Uppsala, Sweden. The HEPES, NaCl, EDTA, Tris and 0.2µM filters were obtained from Fisher Scientific, Springfield, New Jersey. The cysteine was obtained from Aldrich, Milwaukee, Wisconsin.

The carboxymethyl dextran was obtained from Fluka Chemical Corp., Ronkonkoma, New York.

1.2 Peptide design

Each peptide was designed to contain an amino-terminal cysteine residue available for thiol-coupling followed by a KGKG linker to each of the seven hypervariable region (HVR) sequences of adenovirus type 5 hexon. The linker is designed to function as a spacer, as well as to provide a localized positive charge around the cysteine; thus facilitating thiol-coupling by attraction to the negatively charged sensorchip matrix. Each of the seven unique hypervariable sequences corresponding to adenovirus type 5 hexon was obtained from published sequences (Crawford-Miksza and Schnurr, 1996). These amino acid sequences are displayed according to the single letter code in Table 1. All seven linear peptides were synthesized by Research Genetics, Huntsville, Alabama and subsequently purified by HPLC to 85% purity.

1.3 Method of biosensor peptide immobilization

Using the BIACORE 2000™, each of the seven peptides was immobilized onto a flow cell contained on a sensorchip. All peptides were diluted to 500µg/ml. HVR 1, 2, 4, and 5 were diluted with 10mM MES pH 5 and HVR 3, 6 and 7 were diluted in 10mM sodium acetate buffer pH 4. Thiol-coupling chemistry was the standard coupling chemistry used for all peptides. The immobilization procedure was performed at a continuous flow rate of 5µl per minute followed by the sequential injection of 20µl of NHS/EDC, 40µl of PDEA, 50µl of peptide, and 40µl of cysteine/1M NaCl. Each immobilized peptide was subsequently restored with a 5µl pulse of 50mM HCl between sample analyses.

1.4 Biosensor analysis of serum samples:

Interaction of antibodies with Ad5 HVR peptides was monitored using a biosensor instrument (BIAcore 2000™). All BIAcore analysis was performed at 25 °C using 10mM HEPES buffered saline (HBS) with 0.05 % P-20, and 3.4mM EDTA (HBS) as the BIAcore running buffer. Specifically, the BIAcore running buffer consisted of 10mM HEPES, 150mM NaCl, 3.4mM EDTA, and 0.05% P-20 pH 7.4. The HBS was filtered using 0.2µm filter and subsequently de-gassed for 30 minutes at 25°C. Sample diluent contained HBS with 1 mg/ml soluble carboxymethyl dextran (CM-D).

Serum sample analysis was performed by diluting serum 1:20 in HBS w/P-20 and CM-D, filtering through a 0.2µm filter and then testing a 20µl aliquot of each sample for binding to each of the immobilized peptides at a flow rate of 5µl/minute. This protocol was followed for analysis of rats, rabbits and pigs immunized with rAd5, swine samples from a pre-clinical study as well as human samples from a clinical study. In addition, commercial type-specific pAb and mAbs, diluted based on antibody purity (antisera, purified IgG, etc.) were also tested for their reactivity to the HVR peptides. An automated method was run and data collected. The binding of each sample was recorded in response units (RU).

1.5 Affinity purification of anti-HVR1 peptide antibodies

HVR1 peptide was covalently coupled to a Sulfo-link agarose gel (Pierce, Rockford, Illinois). Approximately 2.5 ml of gel was equilibrated with binding buffer (50mM Tris, 5mM EDTA pH 8.5), centrifuged at 500 X g for 5 minutes and supernatant subsequently decanted. A total of 2.8mg of HVR1 peptide was mixed with 2.0ml of gel in a total volume of 2ml buffer for 2 hours at room temperature on a Nutator. (Note: Mixture is light-sensitive, therefore must be covered with foil). The slurry was centrifuged at 500 X g for 5 minutes, and 2 ml of supernatant was removed. The gel was washed 5-6 times with 1 ml aliquots of binding buffer. The gel was then blocked by mixing it with 2 ml of 50mM cysteine for 45 minutes at room temperature. The slurry was centrifuged, liquid decanted, and the gel equilibrated with 4 ml of binding buffer and stored at 4 °C.

A 1.0 ml aliquot of serum from patient IK taken 14 days after receiving rAd5 was mixed with 1.0 ml of phosphate buffered saline (PBS), filtered through a 0.22 µm microcentrifuge tubes. The 2.0ml of diluted serum sample was then added to 0.5 ml of gel and mixed overnight on a nutator at 4°C. The slurry was then transferred to a disposable column. The column containing the gel was then washed with 10 ml of PBS. Bound antibodies were then eluted by addition of 2.0ml aliquots of 100 mM Glycine-HCL pH 2.8 and 0.5ml fractions were collected. Each eluted fraction was neutralized by the addition of 12.5µl of 2M Tris pH 11 to each fraction. All fractions

were then dialyzed against 4 liters of PBS using 10 KD MWCO Slide-a-lyzer cassettes from Pierce. Protein determination of IK (fraction 1) was determined using a Pierce BCA Kit following the manufacturer's instructions.

1.6 Neutralization of anti-peptide HVR1 antibody

In this assay, a constant amount of rAd5 is spiked into diluted serum samples. The spiked serum samples are then added to a monolayer of 293 cells grown on 6 well plates. The "control" consists of 293 cells infected with the rAd5 alone, in the absence of the serum. After a 48 hours infection, the cells are trypsinized, washed and permeabilized, and bound to a FITC labeled antibody to rAd5 hexon. The cell suspension is passed through a Flow Cytometer, and the fraction of fluorescent cells is enumerated. The spiked rAd5 in each sample infects the 293 cells and produce high levels of hexon protein. This protein will bind to the antibody and yield a positive fluorescent signal in the Cytometer. The more infectious virus present in the samples, correlates with a greater # of fluorescing cells. Therefore, a sample containing SNFs to rAd5 will inhibit the infectivity and thus reduce the number of positive fluorescing cells compared to control. A series of dilutions of each serum sample was used and the dilution that gives 50% fluorescence compared to control determines the titer of the SNFs in the serum sample.

1.7 Immune Serum preparation

Rabbits, pigs and rats were immunized with a highly purified, non-replicating rAd5 (Schering-Plough, Kenilworth, N.J.). Two Yorkshire pigs were immunized subcutaneously every four weeks with 200µg of rAd5 in 1.0ml of RIBI adjuvant (RIBI Biologicals) as a primary boost followed by maintenance boosts of 100µg of rAd5 in 1.0ml of RIBI thereafter every month for several years. The six Sprague Dawley rats and two NZW rabbits were immunized subcutaneously every 3 weeks. The rats received a primary boost of 50µg of rAd5 in 0.5ml RIBI followed by maintenance boosts of 10µg rAd5 in 0.5ml RIBI. The rabbits received 25µg of rAd5 in 1.0 ml RIBI boosts throughout. Production bleeds were collected seven days post immunization for all animals. Animals were maintained and procedures preformed by Covance Research Products located in Denver, Pennsylvania.

1.8 Protocol for rAd5 Administration to Pigs and Humans

A total of six immune Yorkshire pigs received multiple subcutaneous and repeated intradermal injections of rAd5 for eight weeks. Cancer patients were administered rAd5 based on the type of cancer and the location of the tumor. All patients received at least 7.5×10^8 particles of rAd5 per dose. Patients with

Hepatocellular Carcinoma were administered a single dose of by percutaneous hepatic artery catheter over a 10 minute period. Patients with malignant head and neck cancer, breast cancer and non-small cell lung cancer received a single intratumoral injection. Cancer patients with peritoneal carcinomatosis from ovarian tumors received a single intraperitoneal instillation over 20 minutes. Serum samples were collected at the indicated time points for analysis.

1.9 ELISA Procedure for Anti-rAd5 Antibodies

Anti-rAd5 antibodies were measured using a sandwich ELISA. Prior to the two-day sandwich ELISA, rAd5 (Schering-Plough, Kenilworth, N.J.) was coated onto microtiter plates overnight and then plates were blocked with bovine serum albumin (BSA). The presence of antibodies directed against rAd5 in pig serum samples or human serum was detected by first diluting the controls and unknown samples 1:40 into phosphate buffered saline (PBS) containing 1% BSA on the plate containing the immobilized rAd5. The samples are then diluted serially 2-fold down the plate. After an overnight incubation, unreacted material was removed by washing the plates and a biotin-labeled Protein A/G conjugate was added for 2 hours and then the unbound conjugate was removed by washing. Horseradish peroxidase (HRP) conjugated streptavidin was then added for 2 hours. The bound HRP-conjugate was quantitatively measured after addition of the enzyme's substrate, TMB (3,3',5,5' tetramethyl benzidine). The amount of antibody against rAd5 is proportional to the intensity of the colored end product of the enzymatic reaction. Samples are considered positive for the presence of antibodies to rAd5 if the mean of the sample O.D. / NPS O.D. is ≥ 0.1 for pig samples (established during assay validation) and O.D. / NHS O.D. is ≥ 0.28 for human samples. In addition, samples were considered positive for development of antibodies after treatment with rAd5 if the mean O.D. of the post-dose sample was ≥ 2 -fold the mean O.D. from the pre-dose sample obtained from the same pig or human serum sample.

2.0 Bioassay for Serum Neutralizing Factors

Serum neutralizing antibodies (SNF) to a rAd5 were determined by using a SaOS-2 anti-proliferation assay. In this assay, the rAd5 vector expresses a p53 protein, a critical checkpoint in cell cycle regulation. The absence or mutation of p53 protein impairs the cell's ability to regulate proliferation resulting in uncontrolled growth. The SaOS-2 cell line (human osteogenic sarcoma) lacks the p53 gene. Infection of SaOS-2 cells with rAd5 expressing p53 results in the inhibition of cell growth. The presence of SNFs will lead to a reduction in the degree of p53-induced inhibition of SaOS-2 proliferation. This is measured as a reduction in anti-proliferation activity of rAd5

spiked into a serum sample resulting in a shift of a dose response curve. The cellular response is monitored using the tetrazolium salt MTT, which is metabolized to formazan, solubilized with sodium dodecyl sulfate (SDS) and then measured spectrophotometrically.

- 5 The observed optical density is related to log cell concentration. Neutralization activity of a serum sample is calculated by comparing the dilution corresponding to 50% maximal inhibition of SaOS-2 cell proliferation of a sample spiked with a standard amount of rAd5 to the dilution corresponding to 50% maximal inhibition of SaOS-2 cell proliferation by a standard amount of rAd5 in the absence of sample. Sample
- 10 results were expressed as % control. Sample results greater than 85 % of control were considered negative for SNF and sample results less than 85 % were reported as positive for SNF.

Table 1. Amino acid sequences of the seven peptides. Each peptide contains a common amino terminus cysteine residue directly followed by a KGKG linker. The remaining amino acid sequences correspond to the seven unique hypervariable regions (HVR) of adenovirus type 5 (Ad5) hexon.

Peptide Designation	Amino acid sequence corresponding to Ad5 hexon	
HVR1	137 CKGKGAATALEINLEEEDDDNEDEVDEQAEQKTHVF	168
HVR2	185 CKGKGIGVEGQTPKYADK	197
HVR3	210 CKGKGYETEINHAAGRVLKK	225
HVR4	247 CKGKGGILVKQQNGKLESQ	260
HVR5	267 CKGKGSTTEATAGNGDNLTPKV	283
HVR6	302 CKGKGMPPTIKEGNSRELMG	315
HVR7	421 CKGKGVINTETLTKVKPKTGQENGWEKDATEFSDK	438

Table 2. Antiserum Reactivity to Immunized Peptides from Pig, Rat, and Rabbit Immunized with rAd5

Animal ID.	Timepoint	Biosensor results in Response units (RU)						
		Pepidat:	HVR1	HVR2	HVR3	HVR4	HVR5	HVR6
Pig								
Ozzie	Pre		3	4		3	3	3
Ozzie (6 months post)	post		456		209	578	212	143
				141				205
RAT								
Normal rat serum (NRS)	NRS		47		44	46	60.5	36
Pooled rat serum from six rats (12 month post)	post		456		76	357	24	87
				105				215
RABBIT								
Normal rabbit serum (NRBS)	NRBS		9		7.5	6.8	5.5	ND
Rabbit 504 (17 months post)	post		754		ND	204	47	92
				115				636*

pre = pre-dosed sample

post = post-dosed sample

ND = Not Determined

D = Day after dosing

* Although there is no data for NRBS, the sample was considered positive due to strong reactivity.

Positive results are indicated in **Bold** based on the following ratio:

RU of post sample / RU of pre/NRS or NRBS sample ≥ 2.0

Table 3. Reactivity of Human Clinical samples from Study C95-178 to Immobilized Peptides

Patient Initials	Timepoint	Peptide:	Biosensor results in Response units (RU)						
			HVR1	HVR2	HVR3	HVR4	HVR5	HVR6	HVR7
ER	PRE		19	19	38.4	14.1	22.1	21.9	28
	D7		ND	ND	ND	ND	ND	ND	ND
	D14		26.2	27.3	41	21.1	28.7	24	31
	D21		20.9	22.3	37	17.1	17.8	21	27
	PRE		23.6	26.9	48.8	23.3	26.1	72.5	29.8
FH	D3		21	21	ND	27	22.5	ND	ND
	D7		38.8	47.1	ND	20.4	15.5	ND	ND
	D14		146.1	34.2	54.6	33.9	36.8	82.7	32.1
	D21		84.8	32.7	47.8	29.3	33.9	68	25
	PRE		24.2	19.9	12.4	12.4	28	17	21.3
IK	PRE		204.6	13.7	ND	41.6	15	ND	ND
	D7		82.66	25.3	37.4	100.6	24.6	17	21.3
	D14		75.49	13	ND	61	9	ND	ND
	D21		83.85	29	38.9	87.3	36.5	18.4	23
	PRE		27.96	10	ND	18	8.5	ND	ND
HH	D28		32.9	20.2	60.8	19.1	22.4	38.1	51
	PRE		30.3	13	ND	13	5	ND	ND
	D3		60.4	22	ND	17.4	18	28.3	42.5
	D7		65.9.6	22.6	44.6	15	9	ND	ND
	D14		6.99	21.5	41.1	17.9	20.4	27.2	30.4
MK	D28		433.3	25.2	ND	9	5	15.2	13.2
	PRE		169.3	14.9	28.1	13.1	10.9	ND	ND
	D3		ND	ND	ND	ND	ND	ND	ND
	D7		14.2	17.6	29.2	15.5	15.9	15.5	13.7
	PRE		23.3	23.3	37.3	18.2	31.5	16	12.0
SM	D6		108	20	28.6	8.6	21	19.5	52.5
	D7		173	12.3	ND	4.4	10.7	19	ND
	D27		150.8	7.4	ND	4.4	10.7	19	ND
	D48								

D = Day

ND = Not Determined

pre = pre-dose

Table 4. Reactivity of Commercial Type-Specific Adenovirus Antibodies to Immobilized Peptides

Vendor	Ab Specificity	Sample I.D.	Biosensor results in Response units (RU)						
			Peptide:						
			HVR1	HVR2	HVR3	HVR4	HVR5	HVR6	HVR7
Biogenesis	Goat anti-Ad2 hexon	post	25	3	17	3	5	15	15
Accurate	Murine anti-Ad penton (65 KD)	post	0	0	0	0	0	0	0
Biodesign	Goat anti-Ad2 hexon	post	30	10	ND	8	13	ND	ND
Biospecific	anti-Ad6	post	ND	ND	16	ND	ND	10	13
Biospecific	anti-Ad3	post	ND	ND	0	ND	ND	0	0
Lee Biol.	Rabbit anti-Ad5	pre	0	0	0	0	0	0	0
		post	255	0	6	0	0	8	10

ND = pre = pre-dosed sample
post = post-dosed sample

ND = Not Determined

Positive results are indicated in **Bold** based on the following criteria:

- Ratio of RU of post sample / RU of pre sample ≥ 2.0 OR
- Post sample (without pre) with a binding greater than 50 RU

Table 5. Reactivity of Pre-Clinical Serum Samples to Immobilized Peptides from Pigs that Received Ad5

Sample I.D.	Timepoint	Peptide:	Biosensor results in Response units (RU)						
			HVR1	HVR2	HVR3	HVR4	HVR5	HVR6	HVR7
377	pre	D15	66	46	101	39	41	87	139
5	pre	D15	1085	56	112	29	58	108	151
384	pre	D15	73	77	89	52	57	98	129
8	pre	D15	749	57	87	131	51	70	102
8	pre	D29	4620	65	81	111	82	71	101
383	pre	D15	96	109	83	83	100	65	80
12	pre	D15	483	127	71	140	57	95	72
12	pre	D29	1490	107	92	201	63	104	99
12	pre	D48	665	113	107	177	94	86	91
389	pre	D15	48	45	73	34	48	69	87
11	pre	D15	729	39	52	32	50	53	64
11	pre	D29	1494	61	62	125	68	58	80
390	pre	D48	1024	66	61	136	65	54	84
11	pre	D15	72	80	74	77	73	60	94
7	pre	D29	936	64	70	62	56	60	81
385	pre	D15	1200	66	69	75	72	58	76
3	pre	D15	567	49	41	41	42	40	55

pre = pre-dose sample

D = Day after dosing

Positive results are indicated in **Bold** based on the following ratio:

RU of post sample / RU of pre sample \geq 2.0

Table 6. Reactivity of Serum Samples to Immobilized Peptides from Patients with Non-Small Cell Lung Cancer Administered rAd5

Patient Initials	Timepoint	Peptide:	Biosensor results in Response units (RU)						
			HVR1	HVR2	HVR3	HVR4	HVR5	HVR6	HVR7
ER	PRE		19	19	38	14	22	ND	28
	D7		ND	ND	ND	ND	ND	ND	ND
	D14		28	27	41	21	29	24	31
	D21		29	22	37	17	18	21	27
	PRE		24	27	50	25	26	73	30
	D7		21	21	ND	27	23	ND	ND
	D14		386	17	ND	20	16	ND	ND
IK	D7		1161	34	55	34	37	83	32
	D21		845	33	48	29	34	68	25
	PRE		24	20	37	12	28	17	ND
	D7		2045	14	ND	42	15	ND	ND
	D14		8266	25	37	101	25	17	21
	D21		7549	13	ND	61	9	ND	ND
	D28		8385	29	39	87	37	18	ND
HH	ZMO.		2796	10	ND	18	22	38	51
	PRE		33	20	ND	19	3	ND	ND
	D3		30	13	ND	13	18	ND	ND
	D7		60	22	ND	22	18	28	43
	D14		680	23	45	17	9	ND	ND
	D21		4839	22	ND	15	20	27	38
	ZMO.		433	25	ND	16	5	ND	ND
MK	PRE		170	15	ND	9	11	15	13
	D3		ND	14	29	13	ND	ND	ND
	D21		11	18	ND	16	16	16	14
	PRE		14	23	37	16	32	16	1230
	D6		108	20	40	14	27	20	1026
	D27		173	12	29	9	21	19	523
	D48		151	7	ND	4	11	ND	ND

D = Day after dosing
 ND = Not Determined
 pre = pre-dose
 MO. = Month's after dosing
 Positive results are indicated in Bold based on the following ratio:
 RU of post sample / RU of pre sample ≥ 2.0

Table 7. Reactivity of Clinical Samples to Immobilized Peptide from Patients Administered rAd5

A. Patients with Recurrent & Malignant Head and Neck Cancer

Patient Initials	Timepoint	Peptide:	HVR1	HVR3	HVR4	HVR7
TGL	PRE		11	32	17	15
	D3		9	24	12	12
	D8		10	29	14	13
	D15		9	27	14	13
	D22		11	31	16	16
	D28		11	29	16	17
RWT	PRE		53	59	27	32
	D3		47	50	23	28
	D10		56	52	26	36
	D14		42	35	17	23

B. Patient with Recurrent Melanoma & Breast Cancer

Patient Initials	Timepoint	Peptide:	HVR1	HVR3	HVR4	HVR7
ES	PRE		27	27	24	20
	D3		23	26	20	18
	D7		54	27	23	19
	D14		252	28	22	19
	D21		185	26	21	18
	D28		79	15	10	8

C. Patient with Peritoneal Carcinomatosis

Patient Initials	Timepoint	Peptide:	HVR1	HVR3	HVR4	HVR7
VKA	PRE		7	35	16	12
	D2		10	33	17	16
	D3		8	30	13	17
	D7		14	49	26	17
	D14		41	36	16	29
	D21		45	44	25	26
	D28		38	31	16	25

D. Patients with Hepatocellular Carcinoma

Patient Initials	Timepoint	Peptide:	HVR1	HVR3	HVR4	HVR7
JT	PRE		50	206	40	132
	D2		10	33	17	16
	D3		25	136	18	85
	D7		1629	109	27	73
	D14		7538	171	108	111
	D21		5208	187	102	116
WMF	D28		3837	174	83	108
	PRE		17	24	20	16
	D3		17	23	20	18
	D7		68	18	17	15
	D14		163	27	22	21
	D28		106	19	18	19
DFB	PRE		12	21	13	14
	D3		14	20	14	18
	D7		49	15	12	16
	D14		1226	26	14	19
	D21		606	22	10	14
	D28		414	32	19	23
RK	PRE		21	21	14	19
	D3		19	18	12	19
	D7		40	25	20	25
	D14		113	21	15	18
	D21		75	19	13	19
	D28		88	32	22	32

PRE = pre-dose

D = Day after dosing

Positive results are indicated in **Bold** based on the following ratio:RU of post sample / RU of pre sample ≥ 2.0

Table 8. Reactivity of pre-clinical swine serum samples from SN 9C380 to Immobilized Peptides

Sample I.D.	Timepoint	Peptide	Biosensor results in Response units (RU)						
			HVR1	HVR2	HVR3	HVR4	HVR5	HVR6	HVR7
377	pre		60.6	46.2	100.8	38.9	41	87.6	138.5
5	D15		50.6	57.6	111.9	79	58	107.6	150.6
384	pre		73.2	71.4	96.9	52.3	57.3	97.6	129.4
8	D28		794.9	56.6	87	130.6	50.7	70	102.3
8	pre		1620	65	81	111.4	62.3	71	101
393	D15		96	199	83.4	83	100.4	95	80
11	D29		482.7	127	71	140	57.2	95	72
12	D48		1490	106.5	92	200.8	63	103.5	90.1
12	pre		664.6	113.2	106.8	176.5	94	86	91
388	D15		47.7	44.6	52	33.5	47.9	69	87
11	D29		728.6	39	52	32	50	52.5	64
11	D48		1494	61.4	62	124.8	68.3	58	80
390	pre		1024	65.6	61	136.2	64.9	54	84
7	D15		71.6	80	73.6	77.3	72.9	60	80
D29			936	64	69.5	62	55.6	56	80.5
385	pre		1200	66.4	69	75	60	58	76
3	D15		106.7	80	65	62	72	59	81
			567.4	49.1	41	41	42	40	55

pre = pre-dose sample

D = Day

Table 9.
C95-177-01- RECURRENT OR METASTATIC HEAD & NECK CANCER

INITIALS	SAMPLE	HVR1	HVR3	HVR4	HVR7
TGL	PRE	11.3	31.5	16.6	15.2
	D3	8.7	23.6	12.1	12.4
	D8	9.5	28.9	14	13.4
	D15	9.2	27	13.6	13.3
	D22	11	30.7	16.3	15.5
	D28	10.9	29.3	15.6	16.6
RWT	PRE	52.5	59	26.6	31.8
	D3	46.8	49.8	23	28.3
	D10	58	52.3	27.9	36.3
	D14	41.5	35	17.4	22.9

I95-082-04 - RECURRENT MELANOMA OR BREAST CANCER

INITIALS	SAMPLE	HVR1	HVR3	HVR4	HVR7
ES	PRE	27	27.4	23.5	19.8
	D3	22.8	25.8	20.3	17.8
	D7	53.7	27.4	23.2	19.4
	D14	252	28.2	21.7	18.6
	D21	185	27.6	20.8	17.7
	D28	78.6	14.6	10	8.2

C95-084-01 - PERITONEAL CARCINOMATOSIS

INITIALS	SAMPLE	HVR1	HVR3	HVR4	HVR7
VKA	PRE	7.4	35	16.2	12
	D2	10.1	33	16.9	15.6
	D3	7.7	29.7	13.1	16.5
	D7	14	48.6	26	17.4
	D14	41	36	16	28.9
	D21	44.5	43.8	25.4	26.1
	D28	38.1	30.6	16.3	24.7

C95-063-01 - HEPATOCELLULAR CARCINOMA

INITIALS	SAMPLE	HVR1	HVR3	HVR4	HVR7
CLL	PRE	8.3	15.9	12.6	11.7
	D3	6.8	17.8	11.8	10.4
JT	PRE	50.3	205.8	39.7	131.7
	D2	10.1	33	16.9	15.6
	D3	24.6	135.6	18	85.1
	D7	1629	108.5	26.6	72.6
	D14	7538	170.6	108	110.6
	D21	5208	186.5	102	116.1
EL	D28	3837	173.8	62.5	107.8
	PRE	23.4	26.6	21.4	22.4
JK	PRE2	21.7	33.7	20.6	33.8
	PRE1	17	28.8	15.9	26.5
WMF	PRE	16.7	23.5	19.5	15.8
	D3	17.3	22.5	19.5	17.5
	D7	67.7	18.3	17	15.3
	D14	163	26.8	22.3	21.4
	D28	106	19.2	17.7	18.9
DFB	PRE	12.3	21	12.6	14
	D3	13.7	20.3	13.9	18.1
	D7	48.7	15.3	11.5	15.6
	D14	1226	26.3	14.3	18.6
	D21	606	22.3	10.4	14.2
	D28	414	32.3	19.1	22.6
RK	PRE	20.7	20.5	14.2	19.2
	D3	18.5	17.5	12.4	19.4
	D7	39.5	24.6	19.5	24.6
	D14	113	21.2	14.9	19
	D21	75.3	19	13.1	19.3
	D28	87.6	31.9	21.9	31.8

Table 10. Results of Anti-/Ad5 Serum Antibody Determined by ELISA, Biosensor and Biosensor Assay from Cancer Patients Administered /Ad5

Cancer Diagnosis	# of Patients	Sample I.D.	Ratio of # Positive Ab / Total # samples			
			ELISA	Biosensor	Biosensor	Biosensor
NSCLC	6	pre	6/6	5/6	0/6	4/6
		post	4/6	1/6		
Melanoma and Breast Cancer	1	pre	0/1	0/1	0/1	0/1
		post	1/1	1/1	1/1	1/1
Malignant Head and Neck Cancer	2	pre	2/2	2/2	0/2	0/2
		post	0/2	0/2		
Peritoneal Carcinomatosis	1	pre	1/1	1/1	0/1	0/1
		post	1/1	0/1	1/1	1/1
Hepatocellular Carcinoma	4	pre	4/4	4/4	0/4	0/4
		post	2/4	0/4		4/4

pre = pre-dosed sample
post = post-dosed sample
ND = Not Determined

Positive results are indicated in **Bold** based on the following criteria:

- 1) Ratio of RU of post sample / RU of pre sample ≥ 2.0 OR
- 2) Post sample (without pre) with a binding greater than 50 RU

Table 11. Summary of Results for Anti-rAd5 Serum Antibody Determined by ELISA, Bioassay and Bioensor Assay from Cancer Patients Administered rAd5

	# Ab Positive / # samples	
	ELISA	Bioensor
Presence of pre-existing Anti-Ad5 Abs	14 / 14	0 / 14
Development of Anti-Ad5 Abs	7 / 14	10 / 14

ELISA Antigen = rAd5
 Bioensor Antigen = specific Ad5 peptides HIV1,3,4, and 7
 Bioassay = rAd5 infection of cells

From the above, it is clear that the present invention provides an efficient method of determining the likelihood that a patient host's immune system will interfere with intended gene therapy using viral vectors.

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SEQUENCE LISTING

SEQ ID NO: 1 is a peptide sequence for the detection of adenovirus
 SEQ ID NO: 2 is a peptide sequence for the detection of adenovirus
 SEQ ID NO: 3 is a peptide sequence for the detection of adenovirus
 SEQ ID NO: 4 is a peptide sequence for the detection of adenovirus
 SEQ ID NO: 5 is a peptide sequence for the detection of adenovirus
 SEQ ID NO: 6 is a peptide sequence for the detection of adenovirus
 SEQ ID NO: 7 is a peptide sequence for the detection of adenovirus
 SEQ ID NO: 8 is a peptide sequence for the detection of adenovirus

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<110> Mytych, Daniel

20

<120> METHODS AND REAGENTS FOR THE DETECTION OF ANTIBODIES TO
 ADENOVIRUS

<130> JB0976

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<170> PatentIn Ver. 2.1

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<212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Peptide for
 detection of adenovirus

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<400> 1

Ala Ala Thr Ala Leu Glu Ile Asn Leu Glu Glu Asp Asp Asn
 1 5 10 15

45

Glu Asp Glu Val Asp Glu Gln Ala Glu Gln Gln Lys Thr His Val Phe
 20 25 30

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<210> 2

<211> 13

<212> PRT

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<213> Artificial Sequence

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<223> Description of Artificial Sequence: Peptide for
detection of adenovirus

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10 <210> 3

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<212> PRT

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detection of adenovirus

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detection of adenovirus

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Val

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<211> 14

60 <212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Peptide for
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<212> PRT

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<223> Description of Artificial Sequence: Peptide for
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Glu Asn Gly Trp Glu Lys Asp Ala Thr Glu Phe Ser Asp Lys
20 25 30

<210> 8

<211> 5

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Peptide for
detection of adenovirus

<400> 8

Cys Lys Gly Lys Gly
1 5

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CLAIMS

We claim:

- 5 1. A method for detecting antibodies capable of binding to adenovirus comprising:
- a) immobilization of a peptide capable of being bound by an anti-adenovirus antibody directly onto a flowcell of a sensorchip in a biosensor,
 - 10 b) obtaining a serum sample from a patient to be tested and contacting said serum sample with the immobilized peptide, and
 - c) measuring binding of antibodies to the immobilized peptide by means of response units from said biosensor.
- 15 2. The method of Claim 1, wherein said peptide is capable of being bound by antibodies specific to adenovirus 5.
3. The method of Claim 2, wherein said serum is human serum.
- 20 4. The method of Claim 3, wherein said peptide is selected from the group consisting of:
- a) a peptide comprising SEQ ID NO: 1, or a peptide having substantial sequence identity thereto;
 - 25 b) a peptide comprising SEQ ID NO: 2, or a peptide having substantial sequence identity thereto;
 - c) a peptide comprising SEQ ID NO: 3, or a peptide having substantial sequence identity thereto;
 - d) a peptide comprising SEQ ID NO: 4, or a peptide having substantial sequence identity thereto;
 - 30 e) a peptide comprising SEQ ID NO: 5, or a peptide having substantial sequence identity thereto;
 - f) a peptide comprising SEQ ID NO: 6, or a peptide having substantial sequence identity thereto; and

- g) a peptide comprising SEQ ID NO: 7, or a peptide having substantial sequence identity thereto.

5. The method of Claim 4, wherein said peptide is selected from the group consisting of:

- a) a peptide comprising SEQ ID NO: 9, or a peptide having substantial sequence identity thereto;
- b) a peptide comprising SEQ ID NO: 10, or a peptide having substantial sequence identity thereto;
- c) a peptide comprising SEQ ID NO: 11, or a peptide having substantial sequence identity thereto;
- d) a peptide comprising SEQ ID NO: 12, or a peptide having substantial sequence identity thereto;
- e) a peptide comprising SEQ ID NO: 13, or a peptide having substantial sequence identity thereto;
- f) a peptide comprising SEQ ID NO: 14, or a peptide having substantial sequence identity thereto; and
- g) a peptide comprising SEQ ID NO: 15, or a peptide having substantial sequence identity thereto.

6. The method of Claim 5, wherein the amount of antibody that binds to each peptide is directly proportional to said response units.

7. The method of Claim 6 wherein said response is detected using a BIACORE 2000™.

8. The method of Claim 1 further comprising a plurality of peptides capable of being bound by an anti-adenovirus antibody are directly immobilized, each on its own separate flowcell.

9. The method of Claim 8, wherein at least one of said plurality of peptides is capable of being bound by antibodies specific to adenovirus 5.

10. The method of Claim 9, wherein said serum is human serum.

11. The method of Claim 10, wherein each of said plurality of peptides are selected from the group consisting of:

- a) a peptide comprising SEQ ID NO: 1, or a peptide having substantial sequence identity thereto;
- b) a peptide comprising SEQ ID NO: 2, or a peptide having substantial sequence identity thereto;
- c) a peptide comprising SEQ ID NO: 3, or a peptide having substantial sequence identity thereto;
- d) a peptide comprising SEQ ID NO: 4, or a peptide having substantial sequence identity thereto;
- e) a peptide comprising SEQ ID NO: 5, or a peptide having substantial sequence identity thereto;
- f) a peptide comprising SEQ ID NO: 6, or a peptide having substantial sequence identity thereto; and
- g) a peptide comprising SEQ ID NO: 7, or a peptide having substantial sequence identity thereto.

12. The method of Claim 11, wherein said peptide is selected from the group consisting of:

- a) a peptide comprising SEQ ID NO: 9, or a peptide having substantial sequence identity thereto;
- b) a peptide comprising SEQ ID NO: 10, or a peptide having substantial sequence identity thereto;
- c) a peptide comprising SEQ ID NO: 11, or a peptide having substantial sequence identity thereto;
- d) a peptide comprising SEQ ID NO: 12, or a peptide having substantial sequence identity thereto;
- e) a peptide comprising SEQ ID NO: 13, or a peptide having substantial sequence identity thereto;
- f) a peptide comprising SEQ ID NO: 14, or a peptide having substantial sequence identity thereto; and

g) a peptide comprising SEQ ID NO: 15, or a peptide having substantial sequence identity thereto.

13. The method of Claim 12 wherein the serum sample is simultaneously
5 contacting with a plurality of immobilized peptides.

14. The method of Claim 13 wherein the amount of antibody that binds to each peptide is directly proportional to said response units.

10 15. The method of Claim 14 wherein said biosensor is a BIACORE 2000™.

16. A method for detecting antibodies capable of binding to adenovirus,
comprising a peptide selected from the group consisting SEQ ID NO: 1, SEQ ID NO:
2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7
15 or a peptides having substantial sequence identity thereto.

17. The method of Claim 16, wherein said method of detecting comprises an ELISA system.

20 18. The method of Claim 17, further comprising a plurality of peptides capable of being bound by an anti-adenovirus antibody.

19. A composition of matter comprising SEQ ID No. 8 or a peptide having substantial sequence identity thereto.

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20. The use of the composition of matter of Claim 19 in a biosensor based assay to detect antibodies.

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ABSTRACT**METHODS AND REAGENTS FOR THE DETECTION
OF ANTIBODIES TO ADENOVIRUS**

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The present invention provides methods and reagents for detecting antibodies to adenovirus. In a preferred embodiment, the present invention is useful for detecting antibodies to adenovirus serotype 5. In a further preferred embodiment, the present invention can be used in a biosensor-based assay. It is contemplated that the present invention is useful to detect antibodies for ascertaining adenovirus infection, evaluating patient response to gene therapy using adenovirus vectors, developing vaccines to adenovirus infection, developing therapeutics for inducing passive immunity to adenovirus infection, as well as other uses.

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 PATENT APPLICATION
 (37 CFR 1.63)**

☒ Declaration Submitted with Initial Filing **OR** ☐ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number JB0976Q

First Named Inventor Mytych

COMPLETE IF KNOWN

Application Number /

Filing Date August 22, 2000

Group Art Unit

Examiner Name

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**METHODS AND REAGENTS FOR THE DETECTION OF ANTIBODIES TO
 ADENOVIRUS**

the specification of which (Title of the Invention)

☒ is attached hereto

OR

☐ was filed on (MM/DD/YYYY) as United States Application Number or PCT International

Application Number and was amended on (MM/DD/YYYY) (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(e) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

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			<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

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[Page 1 of 2]

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Donald W. Wyatt	40879		

☒ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto.

Direct all correspondence to: ☐ Customer Number or Bar Code Label 24265 OR ☒ Correspondence address below

Name	Donald W. Wyatt		
Address	Patent Dept. K-6-1, 1990		
Address	2000 Galloping Hill Road		
City	Kenilworth	State	NJ
ZIP	07033-0530		
Country	USA	Telephone	(908) 298-2902
Fax	(908) 298-5388		

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Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned Inventor

Given Name (first and middle (if any))	Family Name or Surname
Daniel T.	Mytych

Inventor's Signature	Date
Residence: City	Thousand Oaks
State	CA
Country	U.S.A.
Citizenship	U.S.A.
Post Office Address	3162 Saddleback Court
Post Office Address	
City	Thousand Oaks
State	CA
ZIP	91360
Country	U.S.A.

☒ Additional inventors are being named on the 1 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto

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Supplemental Sheet
Page 1 of 1

Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])

Family Name or Surname

Steven J.

Swanson

Inventor's
Signature

Date

Residence: City

Moorpark

State

CA

Country

U.S.A.

Citizenship

U.S.A.

Post Office Address

4318 Timberdale Road

Post Office Address

City

Moorpark

State

CA

ZIP

93021

Country

U.S.A.

Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])

Family Name or Surname

Inventor's
Signature

Date

Residence: City

State

Country

Citizenship

Post Office Address

Post Office Address

City

State

ZIP

Country

Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])

Family Name or Surname

Inventor's
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Residence: City

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Name	Registration Number	Name	Registration Number
Margaret M. Albanese	45525	Susan Lee	30653
Edwin P. Ching	34090	Anita W. Magatti	29825
Nancy V. Connelly	46638	Arthur Mann	35598
Eric S. Dicker	31699	Christine F. Martin	39762
Robert A. Franks	28605	Edward H. Mazer	27573
James M. Gould	33702	Richard B. Murphy	35296
Richard J. Grochala	31518	James R. Nelson	27929
Henry S. Hadad	35888	Pamela G. Salkeld	38607
Thomas D. Hoffman	28221	David B. Schram	43096
Henry C. Jeanette	30856	Immac J. Thampoe	36322
Palaiyur S. Kalyanaraman	34634	Paul A. Thompson	35385
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